Application of a Microfluidic Reactor for Screening Cancer Prodrug Activation Using Silica-Immobilized Nitrobenzene Nitroreductase

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The nitroreductase-catalyzed conversion of a strong electron-withdrawing nitro group to the corresponding electron-donating hydroxylamine is useful in a variety of biotechnological applications. Activation of prodrugs for cancer treatments or antibiotic therapy are the most common applications. Here, we show that a bacterial nitrobenzene nitroreductase (NbzA) from *Pseudomonas pseudoalcaligenes* JS45 activates the dinitrobenzamide cancer prodrug CB1954 and the proantibiotic nitrofurazone. NbzA was purified by affinity chromatography and screened for substrate specificity with respect to prodrug activation. To facilitate screening of alternate potential prodrugs, polyethyleneimine-mediated silica formation was used to immobilize NbzA with high immobilization yields and high loading capacities. Greater than 80% of the NbzA was immobilized, and enzyme activity was significantly more stable than NbzA in solution. The resulting silica-encapsulated NbzA was packed into a microfluidic microreactor that proved suitable for continuous operation using nitrobenzene, CB1954, and the proantibiotic nitrofurazone. The flow-through system provides a rapid and reproducible screening method for determining the NbzA-catalyzed activation of prodrugs and proantibiotics.

Introduction

Nitroreductases have received increasing attention due to their ability to activate prodrugs for in vitro drug therapy. Reduction of a strong electron-withdrawing nitro group to the corresponding electron-donating hydroxylamine results in a very large electronic change, providing an effective enzyme-mediated electronic "trigger".[1] Nitroreductase enzymes, therefore, find significant biotechnological application in the generation of strong cytotoxins by directed enzyme-prodrug therapy (DEPT).[1] A wide range of compounds based on dinitrobenzamide motifs have been synthesized and examined as prodrug candidates. The monofunctional prodrug 5-azirinyl-2,4-dinitrobenzamide (CB1954), for example, can be activated to form a cytotoxic DNA cross-linking hydroxylamine derivative by bacterial nitroreductases.[2−4] DEPT using *Escherichia coli* nitroreductase and CB1954 has been demonstrated to be an effective means to kill cancerous cells[1,5,6] and is in phase 1 clinical trials.[4] For successful application to DEPT therapy, nitroreductases should have high substrate affinity, specificity, and turnover so that sufficient cytotoxin can be generated to kill the tumorous cells. The nitroreductase (NTR) purified from *E. coli* is the most effective and hence extensively studied enzyme for DEPT to date. However, only a very small fraction of the available metabolic diversity of nitroreductases has been explored to date, because screening methods are inefficient. It could be important to screen a wide range of bacterial nitroreductases with the potential ability to be more efficient prodrug activators.

Nitroreductases are also known to activate nitrofuran antibiotics that are used to treat burns, skin grafts, and genitourinary infections.[7,8] The activation of the antibiotics leads to a hydroxylamine intermediate that reacts with DNA and causes its fragmentation.[8] Reduction of nitrofuran antibiotics is critical for their activation, and bacteria with mutations in nitroreductase genes develop nitrofuran resistance.[7,9]

Enzyme immobilization provides a versatile physicochemical tool that allows the reuse or continuous use of enzymes, facilitates substrate and product recovery, prevents product contamination, and in certain instances, improves the properties of the biocatalyst.[10,11] Immobilized enzymes have a significant impact in microfluidic applications,[12] for analytical devices used in peptide mapping[13] and for biocatalytic applications.[14] The entrapment of enzymes on silica-based particles formed through biomimetic silicification reactions has recently been reported as a very efficient method for enzyme immobilization resulting in high thermostability, volumetric activity, and mechanical stability.[15,16] Silica formation in biogenic systems is mediated by cationic proteins and peptides,[17] but a range of simple polyamine molecules[18] can mediate an analogous reaction[15] that combines the advantages of silica encapsulation with a significant reduction in cost. Polyethyleneimine (PEI), for example, precipitates silica in the presence of silicic acid.[19] This water-soluble cationic polymer has been extensively used for stabilizing enzymes by generating hydrophilic microenvironments that protect the enzyme from denaturation.[20,21] However, to our knowledge, there are no reports of silica encapsulation of enzymes using PEI as a mediator.

The nitrobenzene nitroreductase (NbzA) from *Pseudomonas pseudoalcaligenes* JS45 (AC A4468) is able to reduce nitrobenzene to hydroxylaminobenzene.[22] Characterization of NbzA revealed inducible control of NbzA expression by nitrobenzene

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and high affinity for this substrate (low K_m), indicating that nitrobenzene is the physiological substrate for NbzA.[22] NbzA also plays a role during the degradation of 2,4,6-trinitrotoluene by *P. pseudoalcaligenes* JS45, where it catalyzes the reduction of the explosive compound to 2,4-dihydroxylamino-6-nitrotoluene.[23]

Despite the potential pharmacological role of nitroreductases, there are few literature reports demonstrating the immobilization of such enzymes. The majority of the work focuses on biosensing applications.[24,25] The immobilization of a nitroreductase by entrapment in silica particles mediated by PEI is reported herein. The immobilized enzyme preparation was highly stable in a microreactor used as a continuous-flow system to assess nitroreductase activity with nitrobenzene, CB1954, and nitrofurazone.

Experimental Section

Materials. All chemicals were of analytical or HPLC grade and purchased from Sigma-Aldrich (St. Louis, MO). Potassium phosphate buffer (25 mM, pH 7.0) was used throughout unless otherwise indicated.

Expression and Purification of the Nitrobenzene Nitroreductase (NbzA) from *Pseudomonas pseudoalcaligenes* JS45. PCR was used to amplify the *nbz*A gene[26] (682 bp, without the start codon) from *P. pseudoalcaligenes* JS45 genome (G. Zylstra, unpublished data). The primers used were: (forward) 5′-CC**C ATG G**GG *CAT CAT CAC* CAT CAC CAT CCG ACC AGC CCG TTC ATT G−3′ and (reverse) 5′−A**AG CTT** GGC CTA TAC GGA ATT ACC TGG−3′, including the *Nco*I and *Hind*III restriction sites, respectively (bold), and a sequence encoding for six histidine residues (italic). The amplified fragment was cloned into pCR-TOPO vector (TOPO-TA cloning kit, Invitrogen, Carlsbad, CA). After a double digestion by *Nco*I and *Hind*III followed by extraction from an agarose gel, the fragment containing *nbz*A was cloned into the commercial expression vector pBAD-HisA (Invitrogen, Carlsbad, CA), digested with the same enzymes, and designated plasmid pCBJS08. The *Nco*I digestion of pBAD-A released 33 kb from the $(His)_6$ tag, the Xpress epitope, and the enterokinase cleavage site of the vector. The smaller $(His)_6$ tag included on the 5′ primer was used for the purification. The plasmid pCBJS08 was transformed into *E. coli* Top10 (F^- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(ara-leu)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G, Invitrogen, Carlsbad, CA), to give the strain *E. coli* Top10 pBAD-*nbz*A.

An overnight culture from a single colony (1/100, v/v) of *E. coli* Top10 pBAD-*nbz*A was inoculated into Luria broth containing ampicillin (100 microgram/mL). Cultures were grown at 37 °C to an optical density of 0.6−0.8 at 600 nm. L-Arabinose (0.002%) was added, and the cells were incubated at 22 °C for an additional 16 h to induce the expression of the recombinant nitrobenzene nitroreductase.

Cells were harvested via centrifugation (7 000*g*) for 10 min at 4 °C, washed twice, and suspended in saline potassium phosphate buffer (50 mM, pH 7.0, 500 mM NaCl). The cells were lysed by 3 passes through a French pressure cell (16 000 psi). Cell debris and unbroken cells were removed by centrifugation (10 000*g* for 20 min at 4 °C). The supernatant containing heterologous NbzA was loaded on a Co^{2+}NTA affinity column (HiTrap Chelating HP, GE Healthcare, Picataway, NJ) and equilibrated with saline potassium phosphate buffer. After washing with the same buffer, proteins were eluted with a gradient of 500 mM imidazole in saline potassium phosphate buffer. The $(His)_6$-tagged NbzA was eluted with 100 mM imidazole. Fractions with NbzA activity were dialyzed overnight against potassium phosphate buffer at 4 °C, using a Slide-A-Lyzer 10 000 MWCO (Pierce Biotechnology, Rockford, IL).

NbzA Activity. Reductase activity was determined spectrophotometrically (Cary 50 spectrophotometer, Varian Sunnydale, CA) by monitoring the decrease in absorbance at 340 nm due to the oxidation of NADPH. The reaction mixture contained 100 μM nitrobenzene and 250 μM NADPH in potassium phosphate buffer.[22] Assays were performed at 25 °C and continuously mixed with a magnetic stirrer. For the determination of kinetic parameters with nitrobenzene, nitrofurazone, and CB1954, the NbzA activity measurements were performed as described above by varying the substrate concentration in the reaction mixture. One enzyme unit (IU) was defined as the amount of enzyme that catalyzes the oxidation of 1 μmol of NADPH per minute under the specified conditions.

NbzA Immobilization. NbzA immobilization was carried out as previously described by Luckarift et al.[15] Samples (0.5 mL) of enzyme solutions (protein concentration from 0.025 to 2.0 mg/mL) in potassium phosphate buffer (25 mM, pH 8.0) were mixed with 0.125 or 1.25 mL of 10% polyethyleneimine (PEI) (pH 8.0) (final PEI concentrations 1% and 10%, respectively) and 0.125 or 0.375 mL of a hydrolyzed tetramethyl orthosilicate (TMOS) solution (final TMOS concentrations 1% and 3%, respectively). The TMOS was hydrolyzed by dilution in hydrochloric acid (1 mM) to a final concentration of 1 M. The mixture was agitated for 2 min at 22 °C, and the particles were collected by centrifugation for 10 s (14 000*g*) and washed twice in phosphate buffer before use in subsequent experiments. Immobilization yield was defined as the percentage of the initial activity that was immobilized considering the activity remaining in the supernatant. Expressed activity was defined as the ratio of the measured activity of the immobilized enzyme to the difference between the initial activity and the activity in the supernatant.

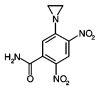
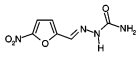
The morphology of the resulting silica particles was characterized by scanning electron microscopy (SEM) (School of Electrical and Computer Engineering, Georgia Institute of Technology, Atlanta, GA).

Stability Experiments. Free enzyme solutions and immobilized enzyme suspensions containing 0.05 IU/mL were incubated in phosphate buffer at 40 °C for thermostability experiments or in the presence of acetonitrile (15%) or methanol (50%) for solvent stability experiments. Samples were withdrawn periodically, and enzyme activity was determined as described above.

Microcolumn Experiments. A stainless steel Microbore column (2 cm × 2 mm, Upchurch Scientific, Oak Harbor, WA) with 0.5 μm frits was filled with a total of 6 IU of silica-encapsulated NbzA (PEI 1%). Packing was performed by sequentially filling the column with samples of a suspension of the silica-encapsulated NbzA pumped through the column using phosphate buffer (5 μL/min). The microcolumn was washed with 5 column volumes of phosphate buffer prior to use. All flow-through experiments were carried out at room temperature (22 °C) using a syringe pump (PHD 2000 infusion, Harvard Apparatus, Natick, MA). To determine the substrate conversion, phosphate buffer containing 100 μM substrate solution (nitrobenzene, CB1954, or nitrofurazone) and 250 μM NADPH was pumped at flow rates ranging from 1 to 5 μL/min. After changes in flow rates, samples were collected and analyzed after 5 column volumes had been pumped through the microreactor. The stability of the microreactor was assayed with a continuous flow of phosphate buffer containing 100 μM nitrobenzene and 250 μM NADPH at a flow rate of 5 μL/min. Periodically, samples of the eluate were collected and analyzed by HPLC to determine the degree of nitrobenzene conversion. Due to the spontaneous degradation of NADPH and in order to maintain saturating conditions for the enzyme, the nitrobenzene solution was made fresh every 8 h during continuous use.

Analytical Methods. Conversions were monitored by HPLC using a Spherisorb C8 column (5U, 250 mm, Alltech, Deerfield, IL) with a mobile phase of acetonitrile and water (containing 0.05% and 0.1% trifluoroacetic acid, respectively). The concentration of acetonitrile was increased from 20% to 60% over 13 min, with a flow rate of 1.5 mL/min. Compounds were monitored by UV detection at a single wavelength of 254 nm. Protein concentration was determined by using a BCA (bicinchonic acid) protein assay reagent kit (Pierce Biotechnology, Rockford, IL) with bovine serum albumin as a standard.

Table 1: K_m Values (μM) of Different Bacterial Nitroreductases

	Soluble NTR	Soluble NTR	Soluble YwrO	Soluble NbzA	PEI silicaencapsulated NbzA (Apparent K_m)
	Escherichia coli [1, 2, 45]	*Enterobacter* [27]	*Bacillus amyloliquefaciens* [3]	*P. pseudoalcaligenes* JS45	
Nitrobenzene 	NT[1]	NT	NT	2.3 (± 0.35)	2.0 (± 0.23)
CB 1954 	862	NT	618	11.7 (± 1)	33.7 (± 4.5)
Nitrofurazone 	64	714	NT	1763 (± 572)	5123 (± 687)

[1] NT; not tested.

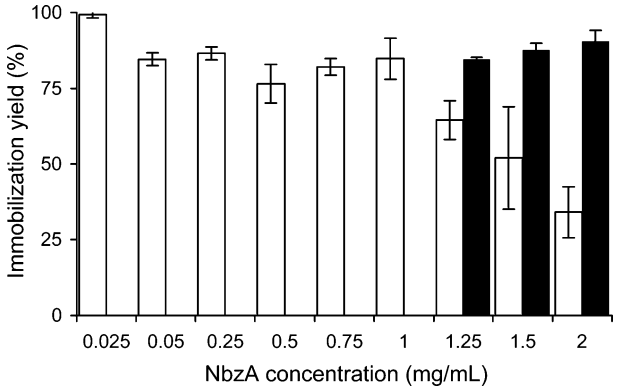


Figure 1. Effect of immobilization efficiency with increasing NbzA concentration. NbzA was immobilized using PEI 1% and TMOS 1% (white bars) or PEI 1% and TMOS 3% (black bars).

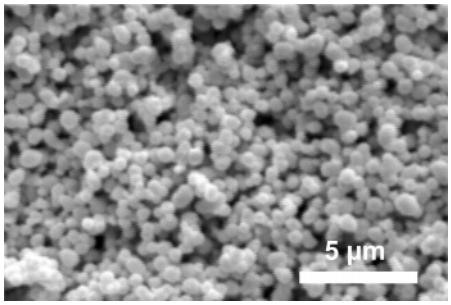


Figure 2. SEM micrograph of the PEI-mediated silica microparticles containing silica-encapsulated NbzA. Samples were sputtered with gold before analysis.

Results and Discussion

NbzA Expression and Purification. The nitrobenzene nitroreductase (NbzA) from *P. pseudoalcaligenes* JS45 was purified from *E. coli* Top10 with a yield of approximately 1 mg purified $(His)_6$-tagged protein per liter of culture. Initial attempts to heterologously express the protein using the 3 kDa purification tag from pBAD-HisA (containing the entire $(His)_6$ tag, Xpress epitope, and enterokinase cleavage site of the vector) led to expression of an inactive protein, presumably due to the additional sequence regions of the vector (data not shown). To clone an active protein, a shorter $(His)_6$ tag was inserted into the 5′ PCR primer, and the purification tag was removed from the pBAD-HisA by *Nco*I enzymatic digestion.

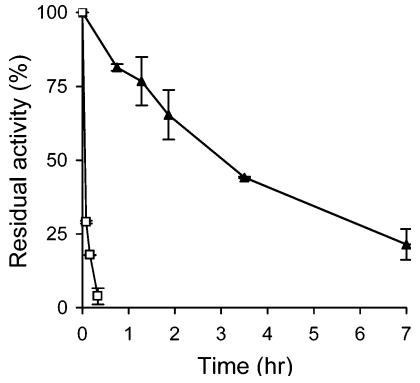


Figure 3. Thermal stability of soluble and PEI silica-encapsulated NbzA. Open squares, soluble NbzA; solid triangles, PEI silica-encapsulated NbzA. The PEI silica immobilized and soluble NbzA preparations contained 0.05 IU/mL and were incubated at 40 °C in potassium phosphate buffer. PEI silica-encapsulated NbzA was prepared with 1% PEI.

The molecular weight of the soluble $(His)_6$ tag heterologous NbzA was 33 kDa, which is consistent with the size of the protein purified from *P. pseudoalcaligenes* JS45.[22] The K_m for nitrobenzene of the $(His)_6$ tag NbzA purified from *E. coli* in this study was 2.3 (± 0.4) μM, consistent with NbzA purified from whole cells of *P. pseudoalcaligenes* (5 μM)[22] (Table 1), indicating that heterologous expression and addition of histidines at the N terminus did not substantially modify the protein activity toward nitrobenzene.

NbzA Immobilization. PEI is an inexpensive polyaminated polymer that has been shown to direct the formation of structured silicas in which the morphology can be manipulated by variations in the reaction conditions.[19] We investigated the immobilization of NbzA by entrapment in a silica matrix deposited from a silicic acid solution using PEI as mediator. The immobilization was stable and highly efficient for enzyme concentrations ranging from 0.03 to 1 mg/mL with immobilization yields higher than 80% (Figure 1). The remaining enzyme activity (less than 20%) was detected in the supernatant and in

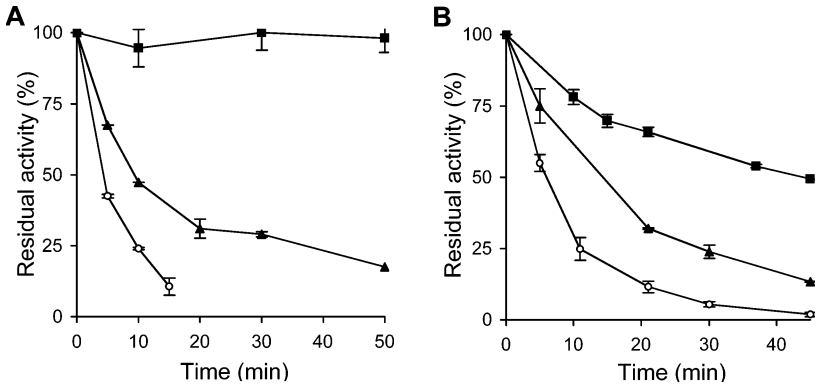


Figure 4. (A) Stability of soluble and PEI silica-encapsulated NbzA in the presence of methanol. Open cirlcles, soluble NbzA; solid triangles, PEI (1%) silica-encapsulated NbzA and solid squares, PEI (10%) silica-encapsulated NbzA. The silica-encapsulated and soluble NbzA preparations contained 0.05 IU/mL and were incubated at 25 °C in potassium phosphate buffer containing methanol (50%) for the indicated times prior to assays. (B) Stability of soluble and PEI silica-encapsulated NbzA in the presence of acetonitrile. Open cirlcles, soluble NbzA; solid triangles, PEI (1%) silica-encapsulated NbzA; and solid squares, PEI (10%) silica-encapsulated NbzA. The silica-encapsulated and soluble NbzA preparations contained 0.05 IU/mL and were incubated at 25 °C in potassium phosphate buffer containing acetonitrile (15%) for the indicated times prior to assays.

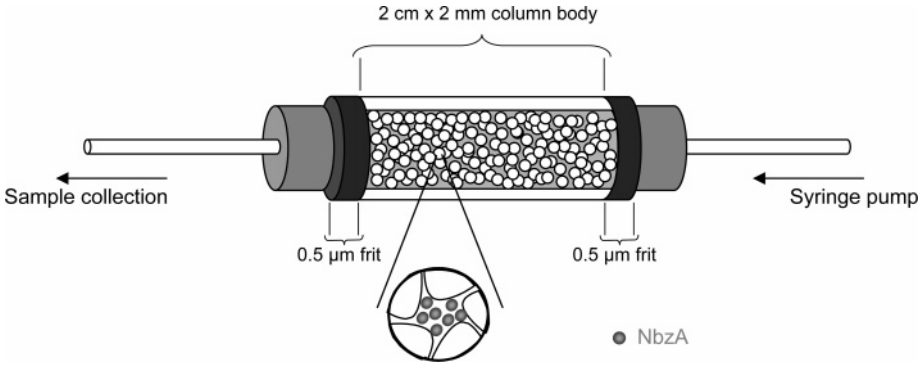


Figure 5. Scheme for immobilized-enzyme microcolumn. The stainless steel body of the microcolumn is 2 mm wide × 2 cm long, for a total volume of 250 μL. 6 IU of silica-encapsulated NbzA in 1% PEI (o) were packed inside the column. Two 0.5 μm frits are placed to both extremities (dark gray). The microcolumn was linked to a syringe pump on one end and a sample collector on the other end.

subsequent wash fractions, indicating negligible loss of activity during immobilization.

The immobilized preparations expressed 60 (±5) % of the theoretical entrapped activity for NbzA concentrations up to 1 mg/mL. Increases in protein concentration above 1 mg/mL resulted in a decrease in immobilization yield as the loading capacity of the silica particles reached saturation, as previously reported.[15] The loading capacity of the reaction could be enhanced, however, by adding additional TMOS to the reaction mixture, thereby extending immobilization yields of higher than 80% to all of the protein concentrations studied (Figure 1).

PEI typically produces silica spheres, but the structures can be manipulated by the addition of solvents during the precipitation reaction, so that the resulting particles can be tailored to suit a specific application.[19] SEM analysis revealed that here PEI mediated the formation of a matrix of interconnected silica particles of approximately 0.5−1.0 μm diameter (Figure 2).

Kinetics Parameters of Silica-Encapsulated NbzA. Nitroreductases from a wide range of organisms differ substantially in their activity against CB1954 and nitrofurazone (Table 1). The desirable properties of potential enzyme-prodrug combinations are a high differential toxicity of the active species relative to the prodrug and a high affinity of the enzyme for the prodrug. The affinity of the soluble NbzA for nitrofurazone is low in comparison to other enzymes,[2,27] whereas the affinity for CB1954 is very high (Table 1). The apparent K_m value for nitrobenzene of PEI silica-encapsulated NbzA was comparable to that of the soluble enzyme (Table 1), indicating that enzyme activity was not significantly hindered by the immobilization within silica particles. For CB1954 and nitrofurazone, apparent K_m values of PEI silica-encapsulated NbzA were 3 and 5 times higher, respectively (Table 1). Diffusion limitations and steric hindrances in immobilized enzyme preparations have been shown to be responsible for higher apparent K_m values compared to the soluble forms.[28−30]

Stability of Silica-Encapsulated NbzA. Enzymes are extremely versatile and able to catalyze a wide variety of chemical reactions, but practical applications are often hindered by the instability of enzymes.[21] The expressed activity of silica-encapsulated NbzA remained unchanged when stored at 4 °C for several months (data not shown). The silica-encapsulated NbzA also was dramatically more thermostable than the soluble enzyme (Figure 3). The enhanced stability of immobilized enzyme preparations provides a greater versatility for their use in a wide range of applications.[14,31−34] The higher thermal stability of the silica-encapsulated NbzA appears to be a consequence not only of the movement constraints imposed by the rigidity of the support[35] but also of the presence of PEI itself, which has proven in many instances to have a protective effect on the stability of enzymes.[20,34,36,37]

A primary limitation of prodrugs such as CB1954 is their relative insolubility in aqueous solutions.[38,39] The encapsulation of the nitroreductase enzyme within silica might protect the enzyme from solvent denaturation and provide an opportunity to screen poorly soluble prodrugs if the compounds are dissolved in cosolvents. Immobilization of NbzA in silica exerted a

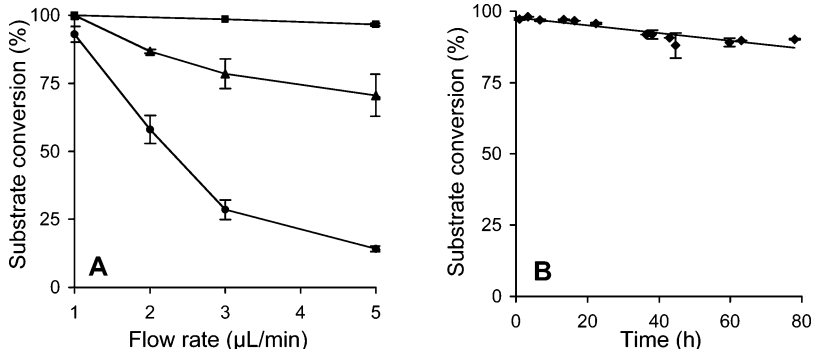


Figure 6. (A) Effect of flow rate on the NbzA column activity. Activity was determined at a range of flow rates between 1 and 5 μL/min. The reaction buffer contained 100 μM substrate (nitrobenzene, squares; CB1954, triangles; and nitrofurazone, circles) and 250 μM NADPH in phosphate buffer. The microcolumn was packed with PEI silica-encapsulated NbzA and contained 6 IU. (B) Operational stability of the NbzA microcolumn. The operational activity was tested with the microcolumn packed with PEI silica-encapsulated NbzA and containing 6 IU. The reaction buffer contained nitrobenzene 100 μM and NADPH 250 μM in potassium phosphate buffer. The continuous flow rate was 5 μL/min.

significant stabilizing effect in methanol and acetonitrile relative to the soluble enzyme (Figure 4). The solvent stability of the immobilized enzymes was even more pronounced when a high concentration of PEI was added during the immobilization process. Addition of PEI concentrations ranging from 1% to 10% to soluble NbzA preparations did not modify the enzymatic activity (data not shown) but provided a protective environment for the enzyme that resulted in complete retention of the initial activity for up to 5 h, in the presence of 50% methanol (Figure 4A). A higher PEI concentration also proved critical for improving the enzyme stability in the presence of acetonitrile (Figure 4B). PEI is a highly hydrophilic molecule; therefore, stabilization could be the result of formation of a hydrophilic shell that promotes a partitioning of the organic solvent, decreasing its concentration in the enzyme microenvironment, an effect that has been previously reported.[40] Thus, by increasing the amount of PEI in the surroundings of the enzyme, we were able to minimize the negative effect that the organic solvent molecules exert on the enzyme stability.

Continuous Flow-Through System. The size and stability of the silica-encapsulated NbzA particles obtained with our method are suitable for flow-through applications. For microfluidic purposes,[13] for example, small particle sizes provide a high surface-to-volume ratio and a concomitant increase in mass transfer efficiency. Silica-encapsulated NbzA was packed into a stainless steel microfluidic device as shown in Figure 5. Throughout all the experiments, no enzyme activity was detected in the eluate, indicating that the NbzA was physically entrapped within the silica particles during silica formation and there was no leaching from the particles during continuous use.

Flow rates typical of microfluidic applications (1−5 μL/min) were used[41,42] without creating back-pressure problems. The flow-through system proved suitable for continuous operation. Conversions of nitrobenzene, CB1954, and nitrofurazone were achieved at a wide range of flow rates (Figure 6A). The conversion decreased with increasing flow rates as expected as a consequence of a reduction in the residence time of the substrate within the microcolumn and the reduced contact time between the substrate and the immobilized enzyme. At 1 μL/min, the three substrates were stoichiometrically converted (Figure 6A). With increasing flow rates, the conversion correlated with the previously measured substrate specificity of NbzA. NbzA, for example, demonstrated the highest affinity for nitrobenzene, and maximum conversion efficiency (substrate conversion per time unit) was achieved at flow rates up to 5 μL/min. The mechanical properties of the immobilized enzyme provided excellent operational stability. A conversion of more than 90% of the nitrobenzene was observed during continuous use for more than 3 days at room temperature and a flow rate of 5 μL/min (Figure 6B).

Other nitroreductases with affinity for CB1954 that have been recently purified and characterized[3,43] could be immobilized in PEI−silica and packed on the microcolumn, as an efficient and cost-effective way to screen for different prodrug reductions. The microreactor designed in this study could also be useful to test the reduction of nitrofurazone derivatives by NbzA or other nitroreductase enzymes immobilized inside the microcolumn.

Conclusion

Advances in the field of microreactors are limited by the availability of new support materials and immobilized enzymes or the lack of robustness of the immobilized preparations.[12,44] On the basis of our results, PEI-mediated silica encapsulation seems to be an effective way to immobilize enzymes for use in microreactors. Moreover, the excellent stability properties of our system provided the basis for a range of potential microfluidic applications of silica-encapsulated enzymes. Further experiments on the tailoring of the physical morphology of the PEI silica for a particular application as well as optimizing the column configuration will broaden the range of applications of the system.

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